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Abstract

Roasting is known to affect the protein profile and allergenicity of hazelnuts (*Corylus avellana* cv TGL). The aim of the study was to investigate whether roasting techniques based on different heat transfer methods (hot air and infrared), differently affect the protein solubility and the IgE-binding capacities of both the soluble and insoluble hazelnut protein fractions. The immune-reactivity of the Cor a 9, Cor a 11 and Cor a 14 allergens resulted to be stable after roasting at 140°C, for both types of treatment, while roasting at 170°C caused a reduction in IgE-binding, which was particularly noticeable after infrared processing, that led to an almost complete disappearance of allergenicity. Microscopical analyses showed that roasting caused cytoplasmic network disruption, with a loss of lipid compartmentalization, as well as an alteration of the structure of the protein bodies and of the cell wall organization.

KEY WORDS: *Corylus avellana***,** hazelnut, proteins, allergens, roasting

Abbreviation list: Coomassie Brilliant Blue (CBB), hot air (HA), high temperature (HT), water-insoluble protein (I), infrared (IR), lithium dodecyl sulfate-PAGE (LDS-PAGE), low temperature (LT), oil body enriched sample (O), oil body (OB), periodic acid-Schiff (PAS), protein body (PB), water-soluble protein (S) and total protein sample (T).

1. INTRODUCTION

The major hazelnut producing countries throughout the world are Turkey and Italy (Klockmann, Reiner, Bachmann, Hackl, & Fischer, 2016). Hazelnuts are marketed, both unshelled and shelled, as raw or roasted, and used as ingredients to obtain a wide variety of products, included baked goods, breakfast cereals, ice creams, various dessert formulations and chocolates (Alasalvar & Shahidi, 2008). Industrial thermal processing is applied to remove the kernel skin, to reduce the moisture and to develop a better aroma and taste (Marzocchi et al., 2017). Roasting also results in an extension of the shelf-life, thanks to the inactivation of enzymes and the eradication of undesirable microorganisms (Özdemir et al., 2001). The control of the temperature and of the moisture distribution is crucial for the design of the thermal process, quality control, and for the choice of appropriate storage and handling practices. The most widely used nut roasting method is the convective heat transfer process, which is performed in a hot air (HA) oven, working either in continuous mode or in batch systems (Perren & Escher, 2007). Another innovative possibility is infrared (IR) heating, which has been used successfully for the dry-roasting and pasteurization of almonds (Yang et al., 2010) and, more recently, for hazelnut roasting (Belviso et al., 2017). Unlike conventional heating mechanisms, in which heat is transferred from the surface to the interior, the main advantage of an IR treatment is that roasting proceeds from the inside of the hazelnut outward, without ventilation, which means that the loss of aroma is minimized (Rastogi, 2012). However, most of the effects of IR roasting on nut quality have only been partially studied. Belviso and coworkers (Belviso et al., 2017) established that a hot air system allowed roasted hazelnuts to be obtained with a lower rupture force and improved oxidative stability, compared to hazelnuts roasted with an IR system. Instead, Binello and coworkers (Binello et al., 2018) found that an IR oven was better at preserving the antioxidant compound content of whole hazelnuts, than the other roasting systems. As far as pastes are concerned, hazelnuts treated in an IR oven at a high temperature showed higher viscosity and density, in addition to a stronger aroma.

69 At a molecular level, thermal treatments lead to changes in the carbohydrates, proteins, fats, vitamins
70 and amino acids, according to the temperature and time settings. Fats can be oxidized, vitamins can
71 be inactivated, oligosaccharides can be decomposed hydrolytically or caramelized, and proteins and
72 amino acids can cross-link with other ingredients (Masthoff et al., 2013). Amino acids and
73 carbohydrates can react, during the Maillard reaction, and give rise to Amadori compounds,
74 (Göncüoğlu Taş & Gökmen, 2017). Moreover, proteins can be affected by denaturation and/or
75 aggregation, thereby causing a reduction in solubility and altering immunochemical recognition by
76 disrupting conformational IgE-binding epitopes or exposing new epitopes that had previously been
77 hidden in the native protein. Downs and coworkers (Downs et al., 2016), when studying the retained
78 IgE reactivity of insoluble and soluble roasted walnut proteins, demonstrated that the more intense
79 the thermal treatment is, the more relevant the decrease in protein solubility and the higher the loss
80 in immunoreactivity. Many authors have tried to clarify the direct effect of thermal processing on
81 food allergenicity, but a general consensus is still lacking (Vanga & Raghavan, 2017).

82 Tree nuts belong to the "big eight" food group (milk, soybean, crustacean shellfish, eggs, fish, tree
83 nuts, wheat, peanuts) that include the foods responsible for almost 90% of human food allergies
84 (CODEX STAN1, 1985). Moreover, hazelnuts are the third food to cause anaphylactic shock in
85 children, after cow's milk and eggs and (Costa, Mafra, Carrapatoso, & Oliveira, 2016). The effect of
86 roasting on hazelnut allergenicity is controversial. Hansen et al. (Hansen et al., 2003) demonstrated a
87 reduction in immune-recognition after processing at 140 °C for 40 min, albeit without any clinical
88 significance, as 29% of the subjects showed allergic symptoms upon consumption of roasted
89 hazelnuts. Worm et al. (Worm et al., 2009) reported that roasting hazelnuts at 144 °C (the time was
90 not specified) resulted in a reduction in immunoreactivity, thus suggesting that roasting might lower
91 the risk of an adverse reaction. However, we have recently demonstrated that oleosins maintain their
92 immunoreactivity after roasting (Nebbia et al., 2019, unpublished results).

93 The objective of this study was to determine whether: (i) the use of different roasting techniques could
94 result in different roasted hazelnuts protein profiles and solubility, and (ii) how these alterations could
95 affect hazelnut allergenicity. To this aim, the capacity of children IgEs to bind both the soluble and
96 insoluble hazelnut protein fractions was investigated before and after roasting with two different
97 techniques: hot air (HA) and infrared (IR).

98 ## 2. MATERIAL AND METHODS

99 ### 2.1 The thermal processing of hazelnuts

100 Raw hazelnut (*Corylus avellana* cv. Tonda Gentile delle Langhe, TGL), were obtained from
101 experimental fields in Cortemilia (Cuneo, Italy) and a pilot-scale roasting experiment was set up at
102 Brovind (Cortemilia, Italy). The moisture content of the raw hazelnuts was 3.56 ± 0.107%. Two pilot-
103 scale ovens, based on different technologies, were used: hot air (HA) and infrared (IR). Two different
104 temperature conditions were chosen for each oven: a low temperature (140 °C, LT) and a high
105 temperature (170 °C, HT). Each drying cycle in the HA oven was set at 25, 30 and 35 min for the LT,
106 and at 10, 13 and 17 min for the HT. The cycle times of the IR oven were set at 10, 12 and 14 min
107 for the LT, and at 6, 8 and 10 min for the HT. The temperature of the ovens was monitored by means
108 of a set of calibrated Pt100 temperature sensors placed in strategic positions inside the thermal process
109 volume of each oven. Aliquots of 1.5 and 4.0 Kg of hazelnuts were used for the HA and IR ovens,
110 respectively, and three separate replicates of the roasting experiments were performed (experimental
111 plan in Fig. 1, panel A). The moisture content of the raw and roasted hazelnuts was assessed
112 immediately after each roasting cycle by means of an Infrared Moisture Analyser (Sartorius, Gottinga,
113 Germany).

114 ### 2.2 Extraction of soluble and insoluble hazelnut protein

115 Soluble and insoluble proteins were extracted from raw and roasted hazelnuts in triplicate. One gram
116 of chopped hazelnuts was defatted three times with 10 ml of hexane; the hexane phase was removed,

at each step, after 30 minutes of shaking in ice. The samples were dried for 30 min in a Speedvac concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany) at room temperature (RT). The defatted hazelnut powder was re-suspended in 1.6 ml of 25mM Na_2HPO_4, 1.5M NaCl (pH 7.5), containing 1 tablet of protease inhibitors (Complete, Roche, Basel, Switzerland). After sonication (4 cycles of 10 sec ON and 10 sec OFF), the samples were shacked for one hour and then centrifuged at 21460xg for 20 min at 4 °C. The supernatants were filtrated through a 45 µm filter and collected as water-Soluble (S) samples. The pellets were extract over night (O/N) with 0.8 ml of 7M urea, 2M thiourea, 20mM Tris-HCl and pH 8.8 (Urea buffer). After centrifugation at 21460xg for 20 min at 4°C, the supernatants were filtrated through a 45 µm filter and collected as water-Insoluble (I) samples. The S and I samples were quantified by means of a Bradford assay (Biorad, Hercules, California).

2.3 Extraction of the oil body associated protein

The oil bodies associated proteins were extracted as described in Nebbia et al. (Nebbia et al., 2019, unpublished results). Briefly, samples of chopped raw and roasted chopped hazelnuts were sonicated in a grinding medium (GM 1: 0.6 M sucrose, 10 mM sodium phosphate, pH 9.5) and filtered using a two-layer gauze. The filtrated sample was centrifuged at 21460xg for 20 min at 4 °C and the pad floating on the top was collected. The oil bodies contained in the floating pad were re-suspended in different buffers: GM2 (GM 1 with the addition of 0.1% Tween 20), GM 3 (GM 2 with the addition of 2M NaCl) and Urea 9 M (pH 11). The oil bodies were centrifuged and collected at a final concentration of 100 mg/ml. In order to remove any oil residues, the samples were incubated with a 2.5 volume of diethyl ether, and precipitated with methanol/chloroform/water, as described by Wessel and Flügge (Wessel & Flügge, 1984). The samples were quantified by means of a Bradford assay (Biorad).

2.4 Lithium dodecyl sulfate-PAGE

Lithium dodecyl sulfate-PAGE (LDS-PAGE) was performed using precast gels (NuPAGE 4-12% Bis–Tris, Invitrogen Life Technologies Ltd., Paisley, UK) in an XCell SureLock Mini-Cell System (Invitrogen), according to the manufacturer's instructions. Each sample was diluted in a NuPage LDS Sample Buffer (Invitrogen), under a non-reducing condition, and loaded in equal volumes. Gels were stained with Colloidal Coomassie Blue (Candiano et al., 2004) and scanned with a ChemiDoc MP System densitometer (Bio-Rad).

2.5 Hazelnut allergic patients

Sera from 16 hazelnut allergic pediatric patients were retrospectively collected from the Paediatric Allergy Unit at the Regina Margherita Childrens' Hospital of Turin (Città della Salute e della Scienza, Turin, Italy). Patients with a convincing clinical history of hazelnut allergy were selected. The collected sera were grouped into five pools, according to ImmunoCAP (ThermoFisher Scientific, Waltham, Massachusetts), considering the hazelnut major allergens (Tab.S1 repository):

- pool A, 4 patients (ID1 to 4) with a positive ImmunoCAP to Cor a 1, Cor a 8, Cor a 9 and Cor a 14;
- pool B,1 patient (ID 5) with a positive ImmunoCAP to Cor a 8, Cor a 9 and Cor a 14;
- pool C, 8 patients (ID 6 to 13) with a positive ImmunoCAP to Cor a 9 and Cor a 14;
- pool D, 3 patients (ID 14 to 16) with a positive ImmunoCAP to Cor a 8;
- pool E, all the patients (ID 1 to 16).

Three sera of non-nut sensitized non-allergic hazelnut consumers were pooled and used as a control. The study was reviewed and approved by the local ethics committee (approval no. 312 prot. no. 22050). All the families gave written informed consent before being enrolled in the study.

2.6 Immunoblotting analysis

The S and I hazelnut extracts were mixed (1:1) for the immunoblotting analysis. After LDS-PAGE, the protein bands were electro-transferred into Nitrocellulose Membranes (0.2 µm) with an XCell II

Blot Module in a transfer buffer (Invitrogen) with 10% methanol (v/v). The membranes were blocked with TBS, containing 0.3% Tween 20, for 30 min and incubated O.N. at 4°C with the patients' sera diluted 1:5 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin). After incubation, the membranes were washed three times with a washing solution (TBS, 0.05% Tween 20) for 10 min, and incubated for 1 hour at RT with an anti-Human IgE antibody (Sera Care Life Sciences Inc., Milford, Massachusetts) 1:5000 diluted in the incubation buffer. The membranes were washed three times and developed with an Alkaline Phosphatase Substrate Kit (Bio-Rad).

2.7 Mass Spectrometry analysis

In gel digestion: bands were reduced in 10 mM DTT/50 mM NH_4HCO_3 for 45 minutes at 56 °C and alkylated in 55 mM IAA/50 mM NH_4HCO_3 for 30 minutes in the dark at RT. The bands were washed and destained three times with ACN 50%/50 mM CH5NO3, pure ACN and again with ACN 50%/50 mM NH_4HCO_3. The samples were dried in a 5301 Eppendorf Concentrator (Eppendorf, Hamburg, Germany) and digested O/N. at 37 °C under shaking with modified porcin trypsin (Promega, Madison, Wisconsin) at 75 ng/µl of 25 mM NH_4HCO_3/10% formic acid.

ESI-Q-TOF: the peptide mixtures were desalted on a Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO) prior to the mass spectrometry analysis. The LC-MS/MS analyses were performed using a micro-LC system Eksigent Technologies (Dublin, California). The stationary phase was a Halo Fused C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 µL/min and at an increasing concentration of solvent B, from 2% to 40%, over 30 minutes. The injection volume was 4.0 µL. The oven temperature was set at 40 °C. The LC system was interfaced with a 5600+ TripleTOF^{TM} system (AB Sciex, Concord, Canada), equipped with a DuoSprayTM Ion Source and CDS (Calibrant Delivery System). The mass spectrometer worked in data dependent acquisition mode (DDA) (Cvijetic et al., 2017; Martinotti et al., 2016).

190 *Protein database search*: the DDA files were searched for, using Mascot v. 2.4 (Matrix Science Inc.,
191 Boston), in the UniProt *Corylus avellana* database. The following parameters were set for the search:
192 an S-carbamidomethyl derivate on cysteine as a fixed modification, oxidation on methionine, Acetyl
193 (N-term), Met-loss (Protein N-term M), as variable modifications and three missed cleavage sites for
194 trypsin digestion. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance at 0.1 Da. Only
195 proteins with at least three peptides and with a peptide score > the peptide identity were considered
196 for identification purposes. The list of the identified proteins is available as Table S2 (repository).

197 **2.8 Microscopical analyses**

198 Small pieces of hazelnut (Fig. S1) were fixed in 2.5 % (v/v) glutaraldehyde in a 10 mM phosphate
199 buffer (PB), pH 7.2, O.N. at 4 °C, rinsed in the same buffer and then post fixed in 1% OsO4 in PB
200 for 1 h, at RT. After rinsing in PB, the pieces were dehydrated in an ethanol series (30, 50, 70, 90 and
201 100%; 10 min each step) at room temperature. The samples were infiltrated in 2:l (v/v)
202 ethanol/London Resin White (EMS, PA _ USA) for 1 h, 1:2 (v/v) ethanol/LRW for 2 h, and 100%
203 LRW overnight at 4 °C, according to Moore et al. (Moore, Swords, Lynch, & Staehelin, 1991). Semi-
204 thin sections (1µm) were stained with 1% toluidine blue to check the quality of the sample, which
205 was established using optical microscopy. Based on these observations, ultra-thin sections (70 nm)
206 were then cut and stained with Uranyl Acetate Substituted (Agar Scientific, Stansted UK) and lead
207 citrate before observation with a Philips CM10 transmission electron microscope.

208 **2.9 Histochemistry**

209 *Carbohydrate staining with a periodic acid Schiff reagent (PAS) treatment:* semi-thin sections (1µm),
210 obtained from the LR White embedded samples, were dipped into 1% (w/v) periodic acid for 30 min,
211 rinsed in water for 5 min and stained in a Schiff reagent for 10 min, in the dark, and then were again
212 rinsed in water for 10 min.

213 *Protein staining with Coomassie Brilliant Blue (CBB)*: semi-thin sections (1µm) were stained at RT
214 for 30 min in a 0.25% (w/v) Coomassie Brilliant Blue R-250 dye in a methanol: acetic acid: water
215 solution (MAW, 5:1:4). Semi-thin sections were rinsed in the MAW solution for 15 min at RT and
216 then rinsed in water for 10 min.

217 *Lipid staining with Nile Red*: hazelnut sections (25 µm) were prepared using a cryostat device (Leica).
218 The sections were incubated with 1 mg/ml of Nile Red in acetone and 1:100 diluted in PBS for 10
219 min at RT in the dark. The sections were mounted in 50% of glycerol. Observations were carried out
220 using a confocal laser-scanning microscope (Leica TCS SP2). The specific yellow-gold fluorescence
221 of lipids was measured at 488/550 nm.

222

223 ## 3. RESULTS AND DISCUSSION

224 Although processing methods generally seem promising to reduce IgE reactivity toward tree nuts
225 proteins, it is still uncertain whether roasting aggravates or mitigates hazelnut allergenicity (Vanga &
226 Raghavan, 2017). In this scenario, we hypothesized that roasting may result in different changes in
227 the structure of hazelnut proteins, which not only depend on the temperature level and cycle duration
228 (time/temperature conditions), but also on the heat application method. We then designed an
229 experiment aimed at obtaining roasted hazelnuts with similar moisture contents, by applying either
230 hot air or infra-red roasting, characterized by different heat transfer methods. The final objective of
231 the study was to obtain new evidences on the effects of roasting on hazelnut allergens and to assess
232 the implications pertaining to the detection of hazelnut allergens and the tolerance induction of
233 hazelnut-allergic subjects. the experiments were designed to compare how two different roasting
234 techniques (with temperature/time condition established in preliminary experiments, Fig 1, panel A)
235 can affect hazelnut protein solubility and allergenicity. A low temperature (LT, 140 °C) protocol was
236 set up to simulate the production of soft aroma and light-colored nuts, while a high temperature (HT,

170 °C) protocol was set up to simulate the production of roasted hazelnut pastes with a strong flavor, color and texture. In order to reach the same final dry matter level, a longer roasting time was needed, using the same process temperature (Fig. 1 panel B). The two combinations of time and temperature that ensured comparable end products, in terms of residual moisture, between the two ovens were: 140 °C for 25 min (HA-LT) and 140 °C for 12 min (IR-LT); 170 °C for 17 min (HA-HT) and 170 °C for 10 min (IR-HT). These four experimental conditions (HA-LT, IR-LT, HA-HT and IR-HT) were considered for all the further protein aggregation, solubility and allergenicity determinations.

We observed a decrease in protein solubility for an increasing roasting temperature, for each oven, as already reported for peanuts (Kopper et al., 2005) and walnuts (Downs et al., 2016) (Fig. 2 panel A). Roasting at 140 °C, in particular for HA processing, resulted in similar water-soluble protein (S) and water-insoluble protein (I) profiles to those of raw hazelnuts. The S/I ratio in the samples processed at 170 °C, with both techniques, was reverted. The water-soluble protein component in the raw hazelnuts, and in the hazelnuts processed at 140 °C accounted for 63-73% of the total protein content, for both systems, while the water-soluble proteins in the samples treated at 170 °C represented only 19-26% (Fig. 2 panel A), regardless of the type of oven.

LDS-PAGE was performed on each sample under non-reducing conditions, in order to highlight any possible protein aggregation phenomena. The same amount of hazelnuts was extracted in order to point out any differences in the protein concentrations and the distribution between the S and I fractions, and between the different considered processing protocols. The protein profile of the HA hazelnuts processed at 140 °C showed the closest protein profile to raw samples, for both the S and I fractions (Fig. 2 panel B). Instead, the most severe treatment, in terms of protein shift from water-soluble to water-insoluble fraction (Fig. 2 panel A), was IR processing at 170 °C. The disappearance of the protein bands in the S lane, for HA-HT and for both IR treatments, was is balanced by the appearance of some smearing at a high molecular weight (MW) in I lane, which probably indicates the presence of aggregated, insoluble protein complexes (Fig. 2 panel B), as already reported for

peanuts by Schmidt et al. (2010) and for walnuts by Downs et al. (2016). The oil body protein extracts were also affected by the heat treatment, as previously reported (Nebbia et al., 2019, unpublished results). Unlike the total protein extract profile, the number of protein bands in the OB associated protein extract increased after roasting. Three new bands, which were barely visible in the raw samples, were detected between 45 and 60 kDa (Fig. 2, panel C). IR processing at 170 °C resulted in the appearance of protein smearing, without any detectable singular bands (Fig. 2, Panel C).

The changes in immune-reactivity induced in hazelnuts after processing were then investigated by conducting immunoblotting experiments with the sera of hazelnut pediatric allergic patients. A sample containing both soluble and insoluble proteins (total protein sample, T, obtained mixing the S and I fractions 1:1) and a sample containing the oil body enriched protein fraction (sample O) were incubated with serum pools of allergic patients for each processing condition (Fig. 3, panel A). The T samples were incubated with 4 pools of serum from allergic patients (A, B, C and D), while the O samples were incubated with a single pool (E) containing the sera of all the patients included in the study (Fig. 3 panel A, Table S1). Our experiment confirmed the findings pertaining to walnuts and peanuts (Downs et al., 2016; Kopper et al., 2005), regarding the effect of temperature on antigen-antibody binding. It was found, for the total protein sample, that the higher the processing temperature was, the lower the overall intensity of the protein bands, and, therefore, the lower the immunoreactivity. As far as the OB enriched proteins are concerned, their immune-reactivity appeared to be affected more by the IR treatment than by the HA treatment, at both of the considered temperatures (Fig. 3).

The raw sample was the most immunoreactive of both the T and O protein samples (Fig. 3 panel B). Both of the processing methods performed at LT resulted in very similar immunoreactive protein patterns (Fig. 3 panel C and E), but these patterns were less intense than those of the raw samples (Fig. 3 panel B). The proteins from hazelnuts processed at 170 °C, although less immune-reactive, conserved a detectable reactivity, especially in HA in the 30 to 65 kDa molecular mass range (Fig. 3

287 panel D). Only the IR treatment at 170 °C resulted in an almost complete disappearance of
288 immunoreactivity (Fig. 3 panel F). This evidence partially confirms what Masthoff (2013) reported,
289 and adds novel information about the times, temperatures and technology settings that allow a
290 reduction in hazelnut immunoreactivity to be achieved. To the best of our knowledge, this is the first
291 experiment that has made a direct comparison of the effects of different roasting conditions on the
292 allergenicity of the same original raw hazelnut sample.

293 An important issue that should be taken into account is that different allergens show different
294 responses to heat processing. The extent of the reduction of single immunoreactivity bands, caused
295 by processing, depends on which allergen is present in the band and whether it is thermo-sensitive or
296 not (Costa et al., 2016). Currently, eleven allergens from hazelnuts are reported in the WHO/IUIS
297 Allergen Nomenclature Sub-committee database. Eight of them have been demonstrated to be
298 involved in food allergies (Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12, Cor a 13, Cor a 14, Cor a
299 15).

300 We found that Cor a 9 and Cor a 11, when subjected to electrophoretic separation in a non-reducing
301 condition, were distributed in different subunits, ranging in mass from 10 to 65 kDa (Table 1), as
302 already demonstrated by Rigby et al (2008). Cor a 9 was identified in all the considered bands, either
303 alone or in association with Cor a 11, Cor a 14 or Cor a 8 (Fig. 3, Table 1). Cor a 9 is a seed storage
304 globulin that belongs to the cupin superfamily, and it is formed by an alkaline and an acidic chain.
305 The polypeptides observed for a low MW mass (from 10 kDa to 40 kDa) are probably highly
306 proteolyzed Cor a 9 subunits, as already pointed out by Rigby et al (2008).

307 Cor a 11, a vicillin-like protein, is a glycosylated storage globulin, which also belongs to the cupin
308 superfamily. According to the observed MW, a mature Cor a 11 subunit is likely to be contained in
309 bands T9 and T10 (around 48 kDa), while the smaller polypeptide around 25 kDa (band T5) could
310 correspond to the subunit that is proteolyzed in the seed. The higher MW bands (from T11 to T14)
311 probably contain the glycosylated form of unprocessed subunits (Rigby et al., 2008).

312 Cor a 14 (2S-albumin) is a small globular protein that is characterized by a 4 disulfide bonds, which
313 belongs to the prolamin superfamily. It was identified in the T2 band with Cor a 9, and, according to
314 the molar fraction data (Table 1), it contributed by 67% to the band.

315 In our experiment, Cor a 9, Cor a 11 and Cor a 14 immunoreactivity overall resulted to be stable after
316 roasting at 140 °C, for both treatment types, while roasting at 170 °C caused a reduction in IgE
317 binding, which was particularly noticeable after IR processing, that led to an almost complete
318 disappearance of reactivity (Fig. 3). Cor a 9, Cor a 11 and Cor a 14 have already been reported as
319 being stable after roasting treatments, although the roasting conditions in the different studies were
320 not comparable (De Leon et al., 2003; Dooper et al., 2008; Müller et al., 2000; Pastorello et al., 2002;
321 Pfeifer et al., 2015; Schocker et al., 2000; Wigotzki, Steinhart, & Paschke, 2000). In particular, it has
322 been demonstrated that the beta-barrel-motif of Cor a 9 plays a key role in the retention of stability
323 after thermal treatment as well as during digestion (Moreno & Clemente, 2008).

324 Cor a 8 is a non-specific lipid transfer protein. We found that Cor a 8, in association with Cor a 9,
325 albeit only in band T3, accounted for 80% of the band, according to the molar fraction. By incubating
326 the extract with the sera of patients with IgEs directed only toward Cor a 8 (Pool D), it was possible
327 to highlight the changes in immunoreactivity of Cor a 8 after thermal processing (Fig. 3, lane D).
328 Roasting at 140 °C, with both methods, caused a reduction in IgEs binding to Cor a 8, although to a
329 greater extent in the IR treated samples (Fig. 3 panel E). The immunoreactivity of Cor a 8 was lost at
330 170 °C, for both conditions (Fig. 3 panel D and F). This observation is in agreement with the results
331 of López et al. (2012), who demonstrated that IgE binding to Cor a 8 was affected to a great extent
332 by high temperatures and wet processing (121 °C and 138 °C in an autoclave, for 15 and 30 min,
333 respectively).

334 As for the OB associated proteins, we observed that immunoreactivity was increased after HA
335 roasting at 140 °C, due to the additional immunoreactivity of newly generated high MW bands, in

336 comparison to raw samples (Fig. 3, panel C). Instead, immunoreactivity was reduced in the remaining
337 treatments, following a IR-LT>HA-HT>IR-HT trend, until it completely disappeared.

338 The results demonstrate that hazelnut proteins IR processed at 170 °C almost completely lost their
339 immunoreactivity, as far as both thermo-labile and thermostable allergens are concerned. Unlike HA
340 roasting, IR roasting results in higher rate of heat transfer taking place from the core to the surface of
341 the nut. IR radiation is an electromagnetic radiation transmitted as a wave and converted into heat,
342 when it impinges on the food surface. It is known that exposure of food to electromagnetic radiation
343 results in changes in electronic, vibrational and rotational states of molecules, including proteins
344 (Rastogi, 2012). We, thus, speculate that the observed difference in immunoreactivity between IR
345 and HA roasted hazelnuts, that were comparable in term of residual moisture, relies on effects of the
346 different direction of heat transfer between the two technologies and on the physical changes induced
347 by IR at protein molecular level.

348 Microscopical observations were then performed with the aim of verifying the impact of roasting on
349 the internal organization of the cells in hazelnut seeds. CBB, PAS and Nile red stains were used to
350 study the distribution of proteins, polysaccharides and storage lipids, respectively. After staining with
351 CBB, protein bodies of different sizes were clearly evident inside the cells of mature seeds (Fig. 4A).
352 These protein bodies have a high number of protein inclusions, varying in size and shape, which
353 appeared as white dots after CBB staining. As reported by Dourado et al. (2003), these inclusions
354 may consist of crystal globoids, protein crystalloids or calcium oxalate crystals. Broad and faint
355 polysaccharide cytoplasmic staining was found after using PAS, while more intense staining was
356 found in the cell walls (Fig. 4B), in agreement with the nature of this structure (Cosgrove, 2005). The
357 same PAS staining pattern was also previously described d in Chilean hazelnuts (*Gevuina avellana*)
358 (Dourado et al., 2003) . The neutral lipids stored in the OBs of oil seeds are mobilized to provide the
359 carbon skeletons and energy necessary for their post-germinative growth (Serrano, Suárez, Olmedilla,
360 Rapoport, & Rodríguez-García, 2008). Here, Nile Red staining shaped the OBs in raw hazelnuts (Fig.

361 4C), as already reported for olive cotyledons (Serrano et al., 2008). The same staining treatment led
362 to a signal on structures that only fill the cell in specific regions of the hazelnut seed, confirming their
363 lipidic nature (Fig. 4, inset). Roasting has already been reported to lead to changes in the
364 microstructure of hazelnuts (Saklar, Ungan, & Katnas, 2003). These authors reported that changes in
365 the microstructure of hazelnuts during a roasting process develop gradually for increasing of air
366 temperatures, air velocities and roasting times (Saklar et al., 2003). Considering the roasted samples
367 (Fig. 4 D-O), CBB staining resulted in a major impact on the protein bodies after treatment with HA-
368 HT and IR-HT. The protein bodies in these samples appeared to have partially lost their original
369 structure, with respect to raw hazelnuts (Fig. 4G and 4M). The changes observed in the protein bodies
370 are in agreement with those observed by Saklar et al. (2003), who reported the presence of swollen,
371 granular and aggregated protein bodies in roasted hazelnuts (165 °C, 1 m/s, 25 min). As shown by
372 the staining with PAS, major difference between the shape of the cell wall was evident after LT and
373 HT (straight *vs* wavy), mainly for the HA process (Fig. 4E and 4H, respectively). Regarding lipid
374 localization, the Nile Red staining is distributed throughout the cells, thus showing that lipids fill up
375 most of the cell cytoplasm. They seemed to be mixed with the protein fraction, with a loss in the
376 compartmentalization of the oil in OBs(Fig. 4F, 4I, 4L and 4O). This is in agreement with a partial
377 damage of the sub-cellular organization, due to cytoplasmic network disruption, already reported
378 during roasting (Saklar et al., 2003). Lipid vesicles attached to the external cell wall of the epidermis
379 were also visible, in particular after the HA-LT treatment (Fig. 4F). Protein bodies (PBs) and OBs
380 were observed, at an electron microscopy level, to occupy most of the cytoplasm of mature seeds,
381 and OBs were found to be directly surrounding the PBs (Fig. 5A and 5B). After roasting, regardless
382 of the type of oven, a loss of sub-cellular organization was evident, with the disappearance of the OBs
383 (Fig. 5C and 5F); as a result of the cytoplasmic network disruption, the lipids had spread all over the
384 cell, as also indicated by the Nile Red staining (Fig. 4). In some cases, e.g. in the HA-HT sample (Fig.
385 5D), the OBs may have fused to form a large lipid droplet, while the protein bodies may have lost
386 their typical features. The protein bodies are clearly evident in the LT processed samples (Fig. 5C

and 5F), in agreement with the optical microscopy observation, while they appear to have lost their original HT structure (Fig. 5D and 5F).

4. Conclusion

Thermal processing has been shown to have allergen-, temperature- and time-specific effects. High temperature roasting, regardless of the heat application method, reduced hazelnut protein immunorecognition by allergic patients, to different extents, for the different allergens, depending on their specific thermal stability. Infrared roasting, in particular when applied at a high temperature, resulted in an almost complete loss of hazelnut immunoreactivity.

Microscopical analyses have shown that both of the considered roasting methods markedly affected cell organization. Roasting caused a disruption of the cytoplasmic network with a loss of the compartmentalization of the lipids in OBs as well as an alteration of the protein bodies and cell wall profile.

In the present work, we were able to obtain processed hazelnuts with modified protein structure, that resulted in reduced IgE binding by sera of hazelnut allergic patients. The obtained results may give an answer to the need of food matrices with reduced allergenicity that are suitable for oral immunotherapy.

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CAPTIONS

Fig. 1. Experimental design (A) and water loss correlation between Hot air (HA) and Infrared (IR) roasting, considering two different temperatures (140 °C and 170 °C) and 3 different processing times for each temperature (B).

Fig. 2. Output of the hazelnut protein extraction. Protein quantification, by means of a Bradford assay, of RAW, HA-LT (Hot Air–Low Temperature), HA-HT (Hot Air–High Temperature), IR-LT (Infrared-Low Temperature) and IR-HT (Infrared-High Temperature) samples. The histograms represent the percentage of Soluble (S) and Insoluble (I) proteins in relation to the total amount of

548 proteins (panel A). LDS-PAGE of the S and I fractions for each condition (panel B). LDS-PAGE of
549 the OB (oil body) associated protein fractions for each condition (panel C). MW: molecular weight.

550 **Fig. 3.** Design of the protein extract analysis (panel A): the RAW (panel B), HA-LT (panel C), HA-
551 HT (panel D), IR-LT (panel E) and IR-HT (panel F) samples considered for the LDS-PAGE and
552 immunoblotting analysis. T: Total protein extract (soluble and insoluble proteins mixed 1:1); O: oil
553 bodies associated protein extract; A, B, C, D: allergic patient pools characterized by different
554 ImmunoCAP patterns (patients with positive IgE ImmunoCAP to Cor a 1, 8, 9 and 14 (A), to Cor a
555 8, 9 and 14 (B), to Cor a 9 and 14 (C), Cor a 8 (D); see Table S1 Repository) Pool E: patient pool
556 pertaining to all the patients in the study. From T1 to T14: protein bands from total protein extracts
557 (T) identified by means of LC-MS/MS. From O1 to O8: protein bands from oil bodies associated
558 protein extracts (O); identified by means of LC-MS/MS. HA: Hot air; IR: Infrared; LT: Low
559 Temperature; HT: High Temperature; MW: molecular weight.

560 **Fig. 4.** Microscopical observations of raw and roasted (HA-LT, HA-HT, IR-LT and IR-HT) hazelnut
561 seeds. (A-D-G-J-M) Coomassie Brilliant blue (CBB) staining of the total proteins; details of the
562 protein body modifications after roasting are shown in the inset. (B-E-H-K-N) PAS staining. The cell
563 walls appear to be stained pink. (C-F-I-L-O) Nile red staining. Localization of the oil bodies (in
564 yellow) by means of confocal laser scanning microscopy; details of a specific lipid-rich region of the
565 hazelnut seeds, marked with asterisks, are shown in the inset; oil bodies are indicated with arrows.
566 Bars = 80 μm in A, B, C, D, E, G, H, I, J, K and 50 μm in F, L, M, O. HA: Hot air; IR: Infrared; LT:
567 Low Temperature; HT: High Temperature.

568 **Fig. 5.** TEM micrographs of the hazelnuts: A-B: raw sample; C: HA-LT sample; D: HA-HT sample
569 E: IR-LT; F: IR-HT. ob: oil bodies; pb: protein bodies, L: large lipid droplet, W: cell wall. Bars = 3
570 μm in A, 1 μm in B, 2 μm in C, D, E, F. HA: Hot air; IR: Infrared; LT: Low Temperature; HT: High
571 Temperature.

572 **Table 1.** Protein identification of the total protein (T) and OB associated protein (O) extracts by

573 means of LC-MS/MS. MW: molecular weight.

574

575 **Table 1**

N° band	Entry	Name	MW experimental/MW theoretical	Protein Score	N° of matching peptides	Protein coverage (%)	Molar fraction (%)
Hazelnut total proteins extract (T)							
T1	AHA36627.1	Cor a 9	8000/59200	465	7	20.4	100
T2	AHA36627.1	Cor a 9	10000/59200	702	8	26.7	32.6
	ACO56333.1	Cor a 14	100000/12600	173	4	23.1	67.3
T3	AHA36627.1	Cor a 9	11000/59200	297	4	8.9	20.60
	4XUW_A	Cor a 8	11000/9926	84	2	29.3	79.4
T4	AHA36627.1	Cor a 9	15000/59200	411	6	19.3	100
T5	AHA36627.1	Cor a 9	22000/59200	4667	14	32.5	95.62
	AAL86739.1	Cor a 11	22000/51110	69	2	11.6	4.38
T6	AHA36627.1	Cor a 9	34000/59200	1248	10	22	100
T7	AHA36627.1	Cor a 9	36000/59200	3242	18	44.4	100
T8	AHA36627.1	Cor a 9	38000/59200	1251	12	37.2	100
T9	AHA36627.1	Cor a 9	45000/59200	2684	13	31.9	66.17
	AAL86739.1	Cor a 11	45000/51110	842	10	27.5	33.83
T10	AHA36627.1	Cor a 9	47000/59200	2871	12	26.8	49.25
	AAL86739.1	Cor a 11	47000/51110	1984	15	35.5	50.75
T11	AHA36627.1	Cor a 9	50000/59200	2490	14	42.2	80.54
	AAL86739.1	Cor a 11	50000/51110	570	7	18.8	19.46
T12	AHA36627.1	Cor a 9	55000/59200	8359	22	56.4	95.17
	AAL86739.1	Cor a 11	55000/51110	319	6	15	4.83
T13	AHA36627.1	Cor a 9	60000/59200	4154	18	47.9	85.48
	AAL86739.1	Cor a 11	60000/5110	310	7	18.8	14.52
T14	AHA36627.1	Cor a 9	130000/59200	2429	14	37	79.6
	AAL86739.1	Cor a 11	130000/51110	623	7	19	20.40
Oil bodies associated proteins (O)							
O1	AAO65960.1	Cor a 13	12000/14723	2822	4	27.9	55.06
	AAO67349.2	Cor a 12	12000/16745	441	3	20.8	44.94
O2	MK737923	Cor a 15	17000/17741	935	7	42.6	70.27
	AAO67349.2	Cor a 12	17000/16745	172	2	23.3	20.71
	AAO65960.1	Cor a 13	17000/14723	179	2	17.9	9.02
O3	MK962827	Caleosin	27000/26912	1446	10	50.6	55.31
	AAO65960.1	Cor a 13	27000/14723	561	3	26.4	10.87
	AAO67349.2	Cor a 12	27000/16745	515	4	25.8	28.47

	AHA36627.1	Cor a 9	27000/59200	481	7	17.1	5.35
O4	AAO67349.2	Cor a 12	31000/16745	531	4	25.8	54.6
	MK737923	Cor a 15	31000/17741	175	3	32	24.6
	AAO65960.1	Cor a 13	31000/14723	114	3	26.4	20.8
O5	MK737923	Cor a 15	36000/17741	704	6	42.6	78.02
	AHA36627.1	Cor a 9	36000/59200	315	6	15.6	10.07
	AAO67349.2	Cor a 12	36000/16745	142	2	12.6	11.90
O6	AHA36627.1	Cor a 9	45000/59200	1843	9	20.2	44.02
	AAL86739.1	Cor a 11	45000/51110	174	5	13.2	24.88
	AAO67349.2	Cor a 12	45000/16745	90	2	12.6	31.10
O7	AHA36627.1	Cor a 9	50000/59200	765	8	17.7	42.86
	AAL86739.1	Cor a 11	50000/51110	166	4	8	21.69
	AAO67349.2	Cor a 12	50000/16745	60	2	15.1	35.45
O8	AHA36627.1	Cor a 9	55000/59200	2701	12	28.6	77.84
	AAL86739.1	Cor a 11	55000/51110	145	4	10.3	22.16

576

577